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The Ability of Chloroquine To Prevent Tat-Induced Cytokine Secretion by Monocytes Is Implicated in Its In Vivo Anti-Human Immunodeficiency Virus Type 1 Activity

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Hydroxychloroquine at 1 μM reduces the load of human immunodeficiency virus type 1 (HIV-1) in patients, whereas chloroquine (CQ) concentrations above 3 μM are required for inhibition of HIV-1 replication in peripheral blood mononuclear cells. Exogenous HIV-1 Tat reaches the cytosol of T cells by using low endosomal pH, and endosome neutralization by CQ prevents Tat from entering and affecting T cells. We show here that 0.6 μM CQ inhibits cytokine secretion induced by Tat in monocytes without affecting lipopolysaccharide-triggered cytokine release. This finding suggests that the in vivo anti-HIV-1 effect of CQ results not from a direct effect on the infected cell but rather from the capacity of CQ to prevent Tat from perturbing the cytokine balance.

Chloroquine (CQ) has been used as an antimalarial drug for years, with a minimum effective concentration in plasma (against *Plasmodium falciparum*) of 0.6 μM (3). Its close relative hydroxy-CQ (HCQ) has also been found to be effective at 1 μM in treating patients infected with human immunodeficiency virus type 1 (HIV-1) (11, 12). The mechanism that enables CQ to decrease the viral load in vivo is not clear. In vitro inhibition of HIV-1 replication in cultured peripheral blood lymphocytes has been observed only at CQ concentrations from 3 to 10 μM (8, 10). This inhibition is associated with alterations in gp120 glycosylation (10). Nevertheless, such high CQ concentrations are toxic to peripheral blood lymphocytes (8) (data not shown).

We concluded that the antiviral effect of 1 μM CQ against HIV-1 infection in patients is unlikely to result from a direct effect on HIV-1 replication in peripheral blood mononuclear cells (PBMCs), which takes place at CQ concentrations above 3 μM. A more CQ-sensitive process, albeit indirectly related to HIV-1 multiplication, should exist in vivo.

The HIV-1 *trans*-activating protein Tat is essential for viral transcript production and HIV-1 replication (4). Some Tat is also secreted by infected cells, and Tat concentrations up to 40 nM have been found in the sera of HIV-1-infected patients (17). It is generally acknowledged that higher titers are likely present in lymphoid tissues, where HIV-1 actively replicates (16, 17). Extracellular Tat can act as a viral toxin triggering, for instance, T-cell apoptosis and the secretion of numerous cytokines by monocytes. Secreted Tat is therefore likely involved in the shift from a TH1-type cellular response toward a TH2-type humoral response, thereby directly contributing to immune dysfunctions during AIDS (1, 9). We recently showed that Tat enters T cells by endocytosis, taking advantage of the low endosomal pH (pH 5 to 6) that enables acid-triggered Tat

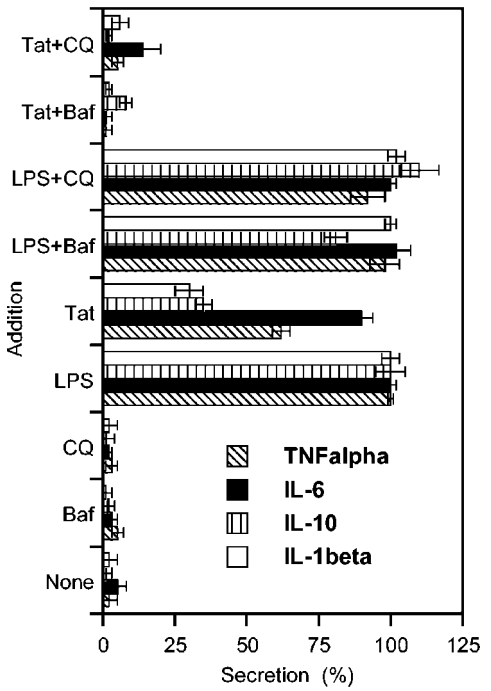

FIG. 1. A submicromolar concentration of chloroquine prevents Tat-induced cytokine secretion by monocytes. Human monocytes were treated with 250 nM Tat or 0.5 μg of LPS/ml. Where indicated, Baf (10 nM) or CQ (0.6 μM) was added 20 min before Tat or LPS. Supernatants were harvested after 4 h (or 24 h for IL-10), and cytokine concentrations were assayed by ELISA. One hundred percent secretion refers to levels obtained using LPS stimulation. These amounts were 3.7, 4.1, 1.3, and 1.5 ng/ml on average for TNF-α, IL-6, IL-1β, and IL-10, respectively.

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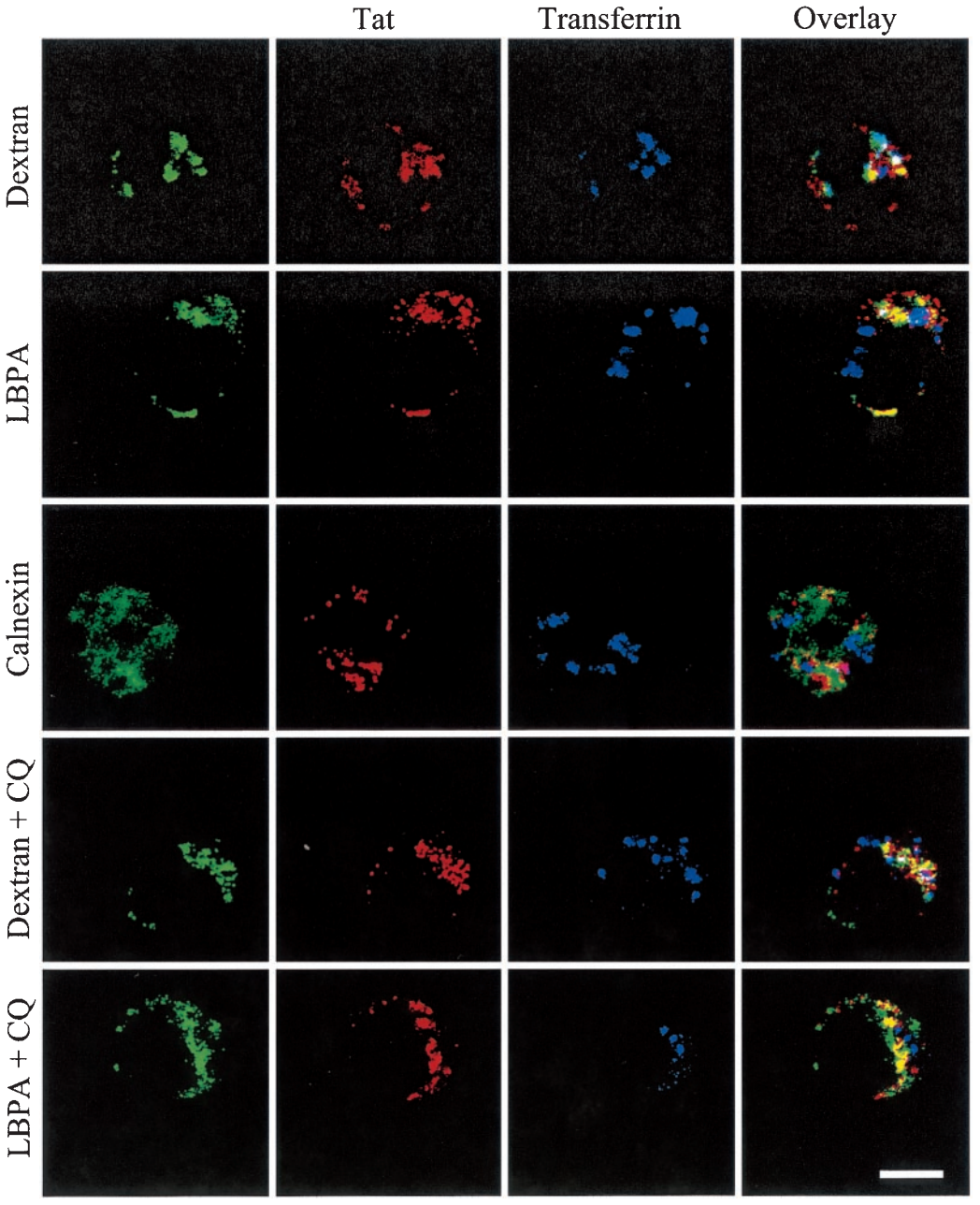

FIG. 2. HIV-1 Tat endocytosis by human monocytes. Cells were labeled for 6 h with Tat, in the presence or absence of dextran-fluorescein to label the lysosomal pathway. Where indicated, 0.6 μM CQ was added 20 min before these tracers. Transferrin-Cy5 was added for the last 45 min to reveal early endosomes. Cells were then washed, fixed, and processed for immunofluorescence detection of Tat and markers of late endosomes/lysosomes (LBPA) or of the endoplasmic reticulum (calnexin), as indicated. Shown are representative median optical sections obtained using a confocal microscope. Bar, 5 μm.

translocation toward the cytosol. Endosomal neutralization using bafilomycin A1 (Baf), a specific inhibitor of the vacuolar proton pump (2), or CQ prevented Tat from entering the cytosol and eliciting cell responses such as apoptosis and cytokine hypersecretion (14). We thought that Tat could be the HIV-1 virulence factor whose most *trans*-cellular activities, such as induction of cytokine secretion, would be inhibited by CQ.

We thus examined Tat-induced production of both proinflammatory (interleukin-1β [IL-1β], IL-6, and tumor necrosis factor alpha [TNF-α] [7]) and immunosuppressive (IL-10 [1]) cytokines by monocytes purified from PBMCs. PBMCs were obtained from human blood (EFS, Montpellier, France) by Ficoll-Paque$^+$ gradient centrifugation and resuspended in RPMI medium containing 10% pooled human AB$^+$ sera. Monocytes were allowed to adhere to plastic tissue culture plates for 3 h and were washed before addition of 250 nM HIV-1 Tat (BH10 isolate, endotoxin free [14]). Similar data,

although showing more donor-to-donor variation, were obtained using 10 nM Tat (data not shown). As a control inducer of cytokine secretion, we used lipopolysaccharide (LPS) (at 0.5 μg/ml), which directly signals from the plasma membrane upon receptor binding (15). Monocyte supernatants were harvested after 4 h of treatment with Tat or LPS for IL-1β, IL-6, and TNF-α assays (7) and after 24 h for the IL-10 assay (1). Cytokine concentrations were determined by enzyme-linked immunosorbent assays (ELISA) (Immunotech). Experiments were performed using blood from six different adult donors; results are means ± standard errors of the means.

LPS-stimulated release of IL-1β, IL-6, IL-10, and TNF-α was not affected by 0.6 μM CQ or 10 nM Baf, whereas these drug concentrations essentially abolished Tat-triggered production of these cytokines (Fig. 1). A submicromolar CQ concentration therefore prevents Tat from affecting T cells (14) and monocytes (Fig. 1). Hence, in PBMCs, Tat-triggered cytokine secretion is a 5- to 15-fold more CQ-sensitive process than HIV-1 replication.

How could CQ prevent Tat-induced cytokine secretion by monocytes? Since CQ did not affect LPS-stimulated release, it probably did not interfere with intracellular signaling or cytokine exocytosis. Hence CQ prevented Tat cytosolic delivery, and we examined whether CQ affected the Tat intracellular pathway in monocytes. To this end, monocytes were plated onto coverslips and then treated for 6 h at 37°C with 100 nM Tat in the presence or absence of dextran-fluorescein (66 kDa; 1 mg/ml) and 0.6 μM CQ, as indicated. Transferrin-Cy5 (100 nM) was added for the last 45 min to label early endosomes. Cells were then washed, fixed, and processed for immunofluorescence detection of Tat and the indicated organelle marker as described elsewhere (14). Anti-lysobisphosphatidic acid (anti-LBPA) was kindly provided by Jean Gruenberg (Geneva, Switzerland), and anti-calnexin was from Becton Dickinson. Samples were viewed under a Leica TCS4D confocal microscope. Upon colabelling with Tat and dextran-fluorescein, both tracers followed the same intracellular pathway and became concentrated within endocytic elements that were devoid of transferrin, presumably late endosomes or lysosomes (Fig. 2). This identification was confirmed by the colocalization of internalized Tat with LBPA, a late endosome/lysosome marker (5), but not with calnexin, an endoplasmic reticulum chaperone. Tat delivery to late endosomes was not affected by 0.6 μM CQ (Fig. 2).

It was important to check, in a functional assay, whether such a minute CQ concentration was indeed able to increase monocyte endosomal pH. We thus examined the capacity of CQ to protect monocytes against *Pseudomonas* exotoxin A. This cytotoxin requires low-pH exposure within endosomes to reach the cytosol, where it inactivates protein synthesis, resulting in cell death (13). Monocytes were treated for 24 h with 100 nM exotoxin in the presence or absence of 10 nM Baf or 0.6 μM CQ before protein synthesis was assayed by monitoring [^{35}S]methionine incorporation (6). Both Baf and CQ significantly protected monocytes against exotoxin A (Fig. 3), indicating that they efficiently raised the endosomal pH.

We concluded that CQ blocks Tat-induced cytokine secretion by monocytes as it does for T cells, i.e., by preventing low-pH-induced Tat endosomal translocation toward the cytosol, a process that we previously documented in T cells and that also involves cytosolic Hsp90 (14).

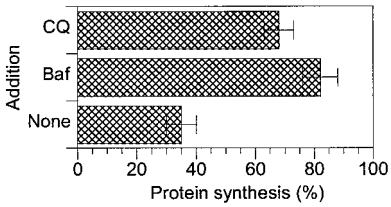

FIG. 3. A submicromolar concentration of chloroquine increases monocyte endosomal pH. Monocytes were treated with *Pseudomonas* exotoxin A, which requires exposure to low endosomal pH in order to reach the cytosol and arrest protein synthesis (13). After 24 h, cell protein synthesis was assayed by using [^{35}S]methionine. Where indicated, 10 nM Baf or 0.6 μM CQ was added 20 min before the toxin. Controls not treated with the toxin were set at 100%.

In a clinical trial to compare the efficacy of 1 μM HCQ and zidovudine in the treatment of HIV-1-infected patients, both treatments were found to be effective, but IL-6 levels were reduced (by 50%) by the HCQ treatment only (11). Interestingly, IL-6 was the cytokine whose secretion was most strongly induced by Tat in monocytes (Fig. 1). LPS-induced cytokine release by monocytes is not inhibited by CQ below 3 μM (15) (data not shown). Hence, the fact that HCQ, but not zidovudine, which directly inhibits virus replication, was able to reduce IL-6 levels in vivo strongly suggests that HCQ decreased the IL-6 level by preventing Tat-induced IL-6 release.

Altogether, these data and the CQ dose-effect relationship discussed above indicate that the ability of HCQ to reduce the virus load in HIV-1-infected patients is likely the result of the inhibition of Tat effects on monocytes and lymphocytes and probably not a direct effect on infected cells. Conversely, the in vivo anti-HIV-1 effect of CQ is further evidence of extracellular Tat involvement in HIV-1 multiplication and AIDS.

Our in vitro observations, together with the results of clinical studies (11, 12), indicate that CQ (or HCQ), at the concentration used for malaria chemoprophylaxis (0.6 μM), could be beneficial against HIV-1 multiplication by fighting extracellular Tat effects. Such an affordable and easily administered drug would be especially useful in the developing world.

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